Journal of Applied Research in Plant Protection 13(2): 123-135 (2024)-*Research Article*

DOI: https://dx.doi.org/10.22034/arpp.2024.17489

Antifungal activity of tobacco Osmotin expressed in *Escherichia coli* against some plant pathogenic fungi

Ali Alizadeh Tilaki, Mostafa Motallebi✉, Zahra Moghaddassi Jahromi, Esmat Jourabchi

Department of Plant Molecular Biotechnology, Institute of Agricultural Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran. ✉motalebi@nigeb.ac.ir

Received: 19 August 2023 Revised: 9 November 2023 Accepted: 22 November 2023

Abstract

The aim of this investigation was to evaluate the antifungal activity of Osmotin against several important economically improtant plant pathogenic fungi. The *Osmotin* gene from *Nicotiana tabacum* was overexpressed in *Escherichia coli* (Rosetta DE3). Taguchi test was applied to optimize the conditions for protein expression, and Western blot analysis confirmed expression of the recombinant protein. The expressed Osmotin was found in the form of insoluble inclusion bodies, which were then solubilized and refolded. The purified and refolded protein's activity was verified by three different antifungal activity assays. The purified recombinant Osmotin demonstrated a wide range of antifungal activity against various types of phytopathogenic fungi: *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Fusarium oxysporum*, *Verticillium dahliae* and *Alternaria brassicola*. Hemolysis biosafety test demonstrated that the protein is non-toxic to mammalian cells. All these findings suggest that the *Osmotin* gene from *N. tabacum* has promising potential as an antifungal agent against various phytopathogenic fungi and also to be utilized for production of antifungal agents and fungal-resistant transgenic plants.

Keywords: Osmotin, PR protein, Prokaryotic expression, Fungi, Recombinant protein

فعالیت ضد قارچی اسموتین توتون بیان شده در *Escherichia coli* علیه برخی قارچ های بیماریزای گیاهی

علی علیزاده تیلکی، مصطفی مطلبی✉، زهرا مقدسی جهرمی، عصمت جورابچی

گروه زیست فناوری مولکولی گیاهی، پژوهشکده زیست فناوری کشاورزی، پژوهشگاه ملی مهندسی ژنتیک و زیست فناوری، تهران، ایران. ✉motalebi@nigeb.ac.ir

دریافت: ۱۴۰۲/۰۵/۲۸ بازنگری: ۱۴۰۲/۰۸/۲۷ پذیرش: ۱۴۰۲/۰۹/۰۱

چکیده

هدف از این تحقیق ارزیابی فعالیت ضدقارچی اسموتین در برابر چندین بیمارگر قارچی مهم گیاهی شامل می‌شد. به منظور انجام این مطالعه، ژن اسموتین از گیاه تنباکو (*Nicotiana tabacum*) در باکتری *Escherichia coli* (Rosetta DE3) همسانه‌سازی و بیان گردید. به منظور بهینه‌سازی شرایط بیان پروتئین، از آزمون تاگوچی استفاده شد. آزمون وسترن بلات بیان پروتئین نوترکیب را مورد تأیید قرار داد. بررسی‌ها نشان داد پروتئین نوترکیب بیان شده عمدتاً به شکل نامحلول اینکلوژن بادی در سلول انباشته می‌شود؛ لذا پس از محلول ساختن این توده‌ها با کمک مواد احیا کننده شرایط ایجاد ساختمان طبیعی تأمین شد. اثر اسموتین نوترکیب بر قارچ‌های *Rhizoctonia solani*، *Botrytis cinerea*، *Sclerotinia sclerotiorum*، *Fusarium oxysporum*، *Verticillium dahleae* و *Alternaria brassicola* مورد بررسی قرار گرفته و فعالیت پروتئین خالص شده از طریق سه آزمون ضدقارچی متفاوت Radial Diffusion، Disk Diffusion و Spore Germination ارزیابی و مشاهده شد که این پروتئین رشد قارچ‌های فوق را مهار می‌کند. همچنین آزمون همولیتیک خونی نشان داد که این پروتئین برای سلول‌های پستانداران فاقد سمیت می‌باشد. یافته‌های فوق نشان می‌دهد که ژن اسموتین گیاه تنباکو دارای توانایی قابل توجهی در مهار رشد قارچ های بیماریزا گیاهی می‌باشد. همچنین این ژن می‌تواند درتولید گیاهان تراریخته مقاوم به قارچ‌های بیماریزا مورد استفاده قرار گیرد.

کلمات کلیدی: اسموتین، PR پروتئین، بیان پروکاریوتی، قارچ، پروتئین نوترکیب

How to cite:

Alizadeh Tilaki A, Motallebi M, Moghaddassi Jahromi Z, Jourabchi E, 2024. Antifungal activity of tobacco Osmotin expressed in *Escherichia coli* against some plant pathogenic fungi. *Journal of Applied Research in Plant Protection* 13 (2): 123–135.

Introduction

Fungal diseases pose a significant challenge to crop production, leading to losses estimated to reach up to 50% in developing countries (Kirubakaran *et al*. 2008). Fungal infections are the cause of about one-third of plant diseases (De Beer & Vivier 2011). Fungal pathogen-induced diseases are regarded as one of the significant or secondary factors responsible for yield losses in numerous important crops (Wang *et al*. 2003). To combat fungal pathogens, plants employ various defense mechanisms, including the production of pathogenesis-related proteins (PRs). These proteins are well studied plant defense proteins that are overexpressed in response to systemic acquired resistance (SAR) mechanisms and pathogen attacks (Kirubakaran *et al*. 2008; Chen *et al*. 2013). One of the function of these proteins is their role as antifungal agents against a wide spectrum of fungal pathogens by increasing plasma membrane permeability and disrupting the membrane potential (Theis & Stahl 2004).

Osmotin is a cationic protein that is widely expressed in plants. It was first identified in tobacco plants, *Nicotiana tabacum* (Huxley), but it has since been found in various other plant species. Osmotin has been studied for its involvement in plant defense mechanisms against both abiotic and biotic stresses (Raghothama *et al*. 1997; Subramanyam *et al*. 2011; Weber *et al*. 2014). One notable characteristic of Osmotin is its molecular weight, which is approximately 26 kilodaltons (kDa), as reported by Singh *et al*. (1987). This size places Osmotin in the range of small to medium-sized proteins. In terms of its amino acid sequence, Osmotin exhibits similarities to several other proteins. For example, it shares sequence similarities with the trypsin/a-amylase inhibitor from maize, thaumatin-like protein, NP24 protein from tomato, antiviral protein gp22, pathogen-related protein C from potato, and tobacco Osmotin-like proteins. These similarities suggest potential functional relationships and shared evolutionary origins between osmotin and these related proteins (Larosa *et al*. 1992). The presence of Osmotin-like proteins in various plant species and their sequence similarities with other functionally diverse proteins highlight the versatile nature of Osmotin and its potential involvement in multiple biological processes, including defense against pathogens and stress tolerance. (Kumar *et al*. 2016; Bashir *et al*. 2020).

It has been demonstrated that pathogens can trigger the expression of Osmotin (Stintzi *et al*. 1991; Woloshuk *et al*. 1991), and expression of this protein has shown to inhibit a spectrum of fungal pathogens (Vigers *et al*. 1992; Liu *et al*. 1994; Abad *et al*. 1996; Annon *et al*. 2014). Meanwhile, since Osmotin or its homologs are non-toxic to humans and exist in edible plants (Singh *et al*. 1987) it is also safe to be used for human consumption.

Despite being discovered early on, the practical applications of Osmotin have been limited due to difficulties in obtaining purified protein. The goal of this research is to use the prokaryotic system for expression and His-tag for purification of Osmotin. This study reports the successful evaluation of the purified Osmotin's antifungal activity against important fungal pathogens isolated from Iran.

Materials and Methods

Plant seeds, fungal and bacterial strains

The seeds for the *N. tabacum* cv. Samsun were obtained from the National Plant Gene Bank of Iran. The study involved the assessment of antifungal activity against several importnat phytopathogenic fungal species, which were sourced from the Iranian Research Institute of Plant Protection in Tehran. These species included *Sclerotinia sclerotiorum* (Lib.) (ABRIICC Ss8), *Botrytis cinerea* (Brown) (ARBIICC Bc2), *Rhizoctonia solani* (Kühn) (ABRIICC Rs46), *Fusarium oxysporum* (Snyder & Hansen) (ABRIICC Fo11), *Verticilium dahliae* (Schwartz) and *Alternaria brassicola* (Ellis) (ABRIICC Ab3). The fungi were cultured and maintained using potato dextrose agar medium, with regular subculturing as necessary. The used *Escherichia coli* (Migula) strains (DH5α and Rosetta DE3) were cultured at 37°C in LB broth. Ampicillin and kanamycin (SIGMA, 100 and 50 mg/ml) respectively, used for selection.

General DNA Extraction

The molecular biology techniques and extraction of genomic DNA from *N. tabacum* Samsun leaves, utilized in this study, were performed in accordance with Sambrook & Russell (2001).

PCR Amplification

The amplification of the *Osmotin* gene was performed using *Pfu* polymerase from Fermentas, with the assistance of two specific primers, GAAGACAACATGGCCACTATCGAGGTCCG as forward primer (ap24N-F) containing *Bpi*I site and TCGAGACCATTAGGACAAAAGATAAC as reverse primer (ap24N-R) containing *Xho*I site. The restriction sites in the primers are underlined within the primer sequences. After purifying the amplified fragment using a recovery kit from iNtRON Biotechnology, the fragment was inserted into the pJET1.2 cloning vector (Fermantas, Germany). The fragment was further subcloned into the pET26b (+) expression vector (Novagen, Germany) at the *Nco*I/*Xho*I restriction sites in a way that it was in frame, driven by T7 promoter and carried a His-tag. The processes of cloning were performed in accordance with Sambrook & Russell (2001). The sequencing confirmed the process, and the final construct was designated as pEAA-2. The pEAA-2 was transformed into the *E. coli* strain Rosetta DE3.

Computer analysis and sequencing

The osmotin sequences were retrieved from GenBank and along with the sequences obtained in this study used for multiple sequence alignment (ClustalW). The *Osmotin* gene cloned in the pEAA-2 recombinant construct was sequenced by Macrogen, South Korea.

Expression in E. coli

The transformed bacterial cells were cultured at 37°C in a 2×TY medium consisting of 16 g/L of bacto-tryptone, 10 g/L of yeast extract, and 5 g/L of NaCl, supplemented with 50 µg/mL of kanamycin and 1% glucose (w/v). When the cells reached an optical density (OD_{600}) of 0.8, they were washed twice to remove the glucose. The Taguchi method was employed to determine the optimal conditions for protein expression as previously reported by Zandvakili *et al*. (2017). The aim of this method is to maximize protein production in *E. coli* by using three factors that affect the expression of foreign proteins (Larentis *et al*. 2014; Papaneophytou & Kontopidis 2014; Silaban *et al*. 2018). Different levels of each factors (Table 1) and all the procedures for production of this protein were adapted as described previously (Zandvakili *et al*. 2017).

Protein extraction, purification and western blotting analysis

These experiments were achieved as described previously (Zebardast *et al*. 2015). The method described by Machuca and Roujeinikova (2017) was used for denaturation and refolding of recombinant protein as inclusion body using urea.

Antifungal activity

To evaluate the antifungal properties of Osmotin, three assays were employed to assess its ability to inhibit fungal growth: radial diffusion, spore germination, and disk diffusion.

Radial diffusion assay: to test the antifungal activity of the purified Osmotin protein, the experiment was achieved based on the reported method with some modifications (Brogue *et al*. 1991). The protein was added to five mm holes in the PDA plates in concentrations of 3, 15, and 30 µg. The plates were kept in an incubator set to 28°C until the mycelial growth had completely surrounded the negative control, and inhibition zone had formed around the holes with Osmotin protein. The growth of fungal species, including *B. cinerea*, *F. oxysporum*, *S. sclerotiorum*, *V. dahliae*, *A. solani*, and *R. solani*, was monitored.

Disc diffusion assay

The antifungal activity of Osmotin against fungal species (*F. oxysporum*, *A. solani*, *B. cinerea*, and *V. dahliae*) was evaluated using a modified method described by Nweze *et al.* (2010). Paper discs were layed on potato dextrose agar plates and treated with a mixture of different concentrations of purified Osmotin (1, 6, 12 µg) and 2×10^4 spore

cells per mL suspension of each of the four fungal species. The plates were kept at a temperature of 28°C until the spores had germinated and the mycelium had started to grow in the negative control discs.

Spore germination assay

Spore germination assay was carried out according to Ziaei *et al.* (2016) by using 15µg of the purified Osmotin as the sample. The inhibitory effect of Osmotin on germination was investigated against *B. cinerea, S. sclerotiorum, F. oxysporum, V. dahlia* and *A. solani*.

Hemolytic assay

The hemolytic activity of recombinant Osmotin was tested against human erythrocytes, as previous reports have indicated that antimicrobial peptides can display cytotoxicity against eukaryotic cells. The Osmotin hemolytic activity was measured using a modified version of the method described by Xu *et al.* (2019). The negative control was human red blood cells in PBS (A_{blank}), and the positive control was human red blood cells in 0.1% (v/v) Triton X-100 (A_{triton}). The hemolytic activity of the negative control was quantified and compared to the Osmotin activity, followed by t-test analysis and calculation of the percentage of hemolysis using this formula:

$$\%Hemolysis = [(A_{sampel} - A_{blank})/(A_{triton} - A_{blank})] \times 100.$$

Statistical analysis

The statistical differences were assessed based on the ANOVA (analysis of variance) using SPSS Software (Ver. 15, USA). Differences were considered significant at a probability level of $P<0.05$. Mean values were compared using least significant different and Duncan tests.

Results

The goal of this research was to assess the antifungal activity of the Osmotin from *N. tabacum* cv. Samsun. The coding region of the gene was amplified from genomic DNA as template and specific primers, ap24N-F/ap24N-R, which had *Bpi*I and *Xho*I restriction sites at their 5' ends, respectively. These primers are designed to amplify sequence that does not have the original signal peptide at the beginning and the carboxyl sequence at the end of the gene.

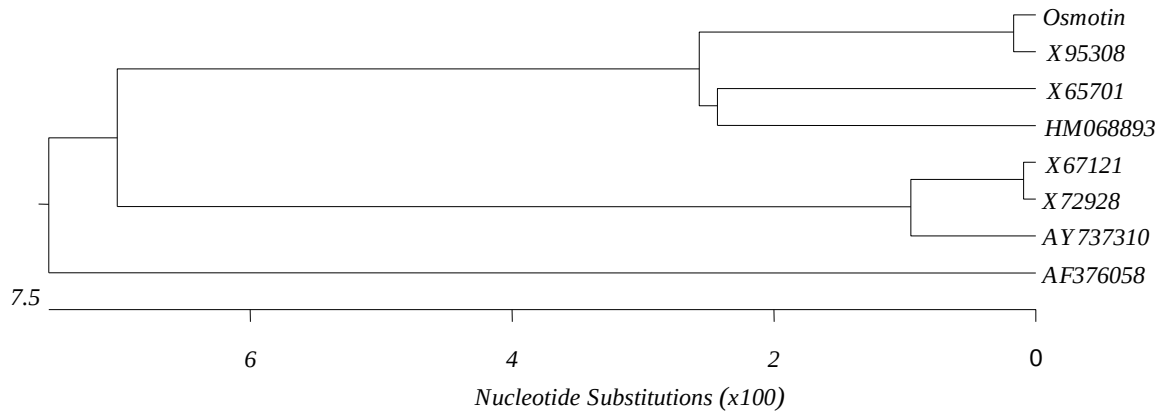


Figure 1. Phylogenetic tree obtained using ClustalW2 by MegAlign at DNASTAR to compare the osmotin DNA sequence with the seven related sequences with accession numbers: X95308, X65701, HM068893, X67121, X72928, AY737310, AF376058. The highest sequence homology belongs to X95308 accession numbers sequence(99.7%) in which there are two nucleotide differences in two codons that did not change amino acid in the protein. Other sequences show lower homology with this sequence which lead to change in protein sequences.

The amplified fragment was cloned in pJET1.2 cloning vector and confirmed by sequencing (Date not shown). Using ClustalW2, multiple sequence alignment of this sequence with the related

sequences (with accession numbers: X95308, X65701, HM068893, X67121, X72928, AY737310, AF376058) was performed and the phylogenetic tree obtained showed very high sequence homology (Figure 1). The highest sequence homology belong to X95308 accession numbers sequence(99.7%) in which there are two nucleotide differences in two codons (third base change) that did not change amino acid in the protein.

Prokaryotic expression

In order to express Osmotin in *E. coli*, the gene's coding sequence was subcloned into the pET26b (+) vector, which had an inbuilt His-tag at the C-terminal end to aid in purification and the new construct was designated as pEAA-2 (Figure 2A). The protein was overexpressed in *E. coli* Rosetta (DE3), which provided tRNA genes for rare codons. The SDS-PAGE and Western blot analysis confirmed successful expression of the recombinant protein (Figures 2B and 2C).

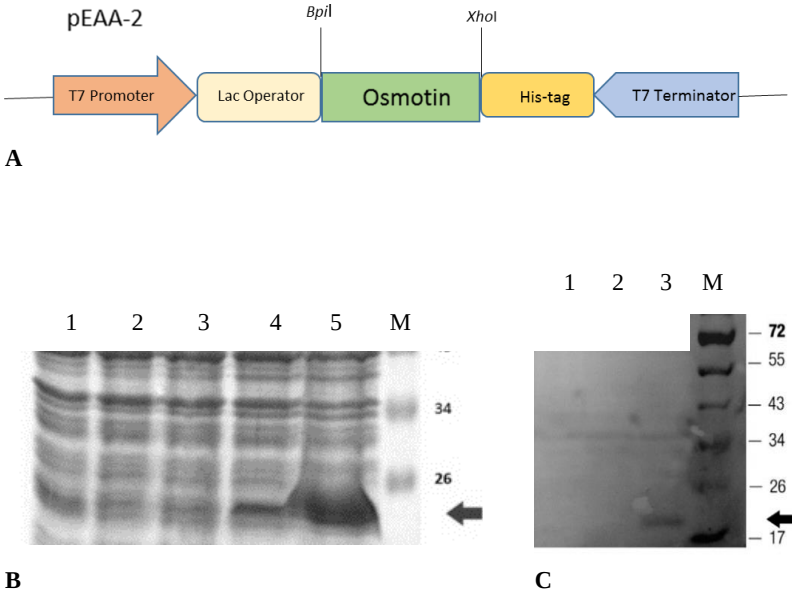


Figure 2. SDS-PAGE and western blot analysis of recombinant protein induction. A: Schematic representation of cassett containing *osmotin* gene in pEAA-2 construct, B: *Escherichia coli* Rozetta (DE3) cells transformed with the pET26b (+) vector containing the *osmotin* gene were grown until they reached an OD of 0.6-0.8 and induced with IPTG. Total protein extracted from the harvested cells and analyzed on a 15% SDS-PAGE. **1**: extraction from non-induced cells, **2**, **3**, **4**, **5**: represent the induced protein expression after 30, 60, 90 minutes and 16 hours, respectively. M: Protein molecular marker, C: Western blot of the recombinant protein using anti-His antibody. **1**: Empty vector, **2**: Non-induced protein extraction, **3**: Induced protein expression, M= Protein molecular marker (kD).

To optimize the expression of the Osmotin protein, the Taguchi method was used with the M16 orthogonal experimental design. The impact of induction time, IPTG concentration and temperature, were analyzed at four levels for each factor (Table 1).

Table 1. Variable factors and levels employed in Taguchi method.

Factors	Level 1	Level 2	Level 3	Level 4
IPTG (mM)	0.2	0.5	0.7	1
Time (h)	2	4	6	16
Temp (°C)	23	28	33	37

A 25.4 kDa protein band was detected on SDS-PAGE, and the amount of the expressed protein was calculated through densitometry by Qualitek-4 software. The density ratio of the expressed protein

to the total protein density was used as the expression index (Figure 3).

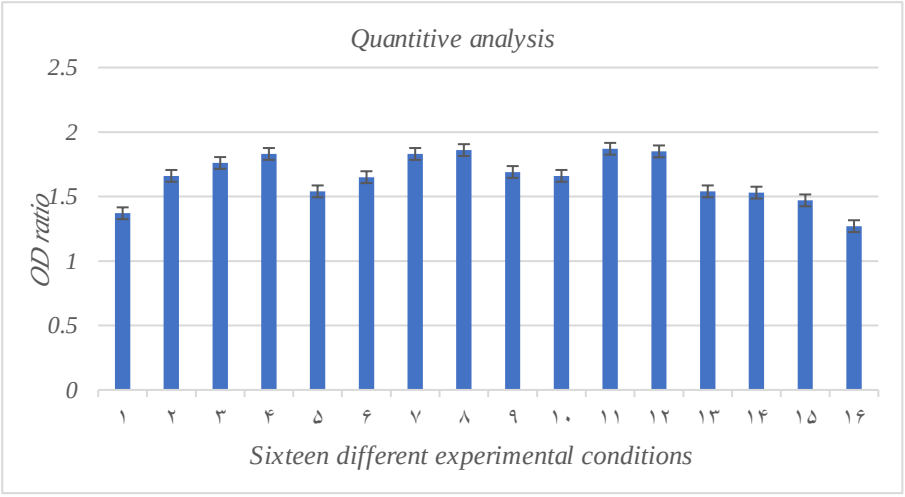


Figure 3. Quantitative analysis of protein expression: 1-16 = Different trials used in 16 experimental design based on taguchi method (Zandvakili *et al.* 2017).

The results indicated that for protein expression the optimum conditions were 33°C, 0.7 mM IPTG concentration, and six hours of induction time. The majority of the expressed Osmotin was precipitated as insoluble protein (inclusion bodies) (Figure 4).

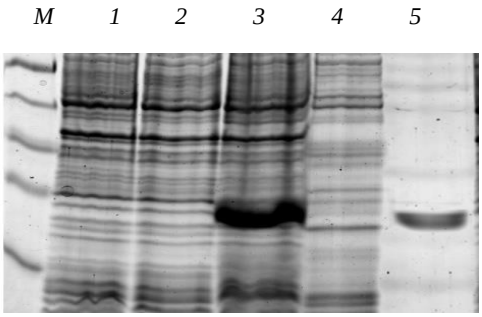


Figure 4. Protein localization, protein extracted from. **1**: *Escherichia coli* cells transformed with empty vector, **2**: non-induced cells, **3**: total protein from induced *E. coli* carrying Osmotin, **4**: soluble proteins and **5**: insoluble proteins (inclusion bodies), M: Protein molecular marker (kD).

The negative control was the non-induced culture. The inclusion body was denatured with 8M urea and refolding was achieved through gradual urea removal via dialysis. The purified refolded recombinant Osmotin was obtained through affinity chromatography using Ni-NTA column (Figure 5). The antifungal activity of the refolded purified Osmotin was approved against some phytopathogenic fungi.

Antifungal activity assay

The antifungal activity of the purified and refolded expressed Osmotin was evaluated *in vitro* using three different tests: radial diffusion, disc diffusion, and spore germination assays. The radial diffusion assay showed that Osmotin protein had an inhibitory effect on the growth of various important plant pathogenic fungi, including *R. solani*, *B. cinerea*, *S. sclerotiorum*, *F. oxysporum*, *V. dahliae*, and *A. brassicola*, at different concentrations (3, 15, and 30 µg) of the protein. The negative controls were elution buffer and extracted protein from non-induced *E. coli* containing *Osmotin* gene in which the fungi were not inhibited and grew over the holes (Figure 6).

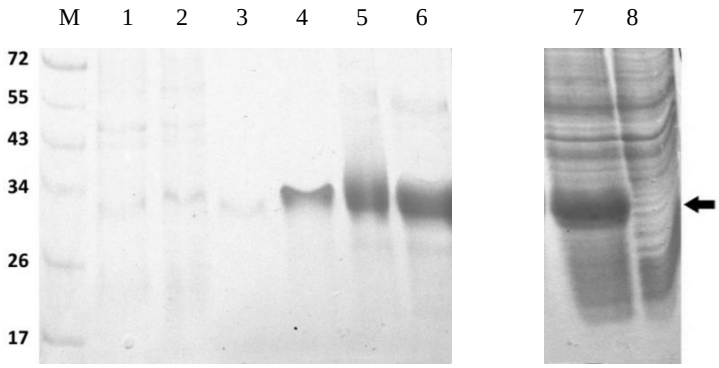
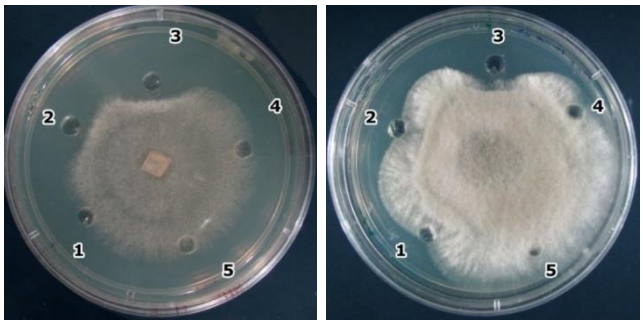


Figure 5. Purified recombinant Osmotin.1: Flow through, 2, 3: Wash, 4, 5, 6: Elution, 7: Total protein from induced *Escherichia coli* carrying Osmotin, 8: Non-induced cells.

A

R. solani



B. cinerea

B

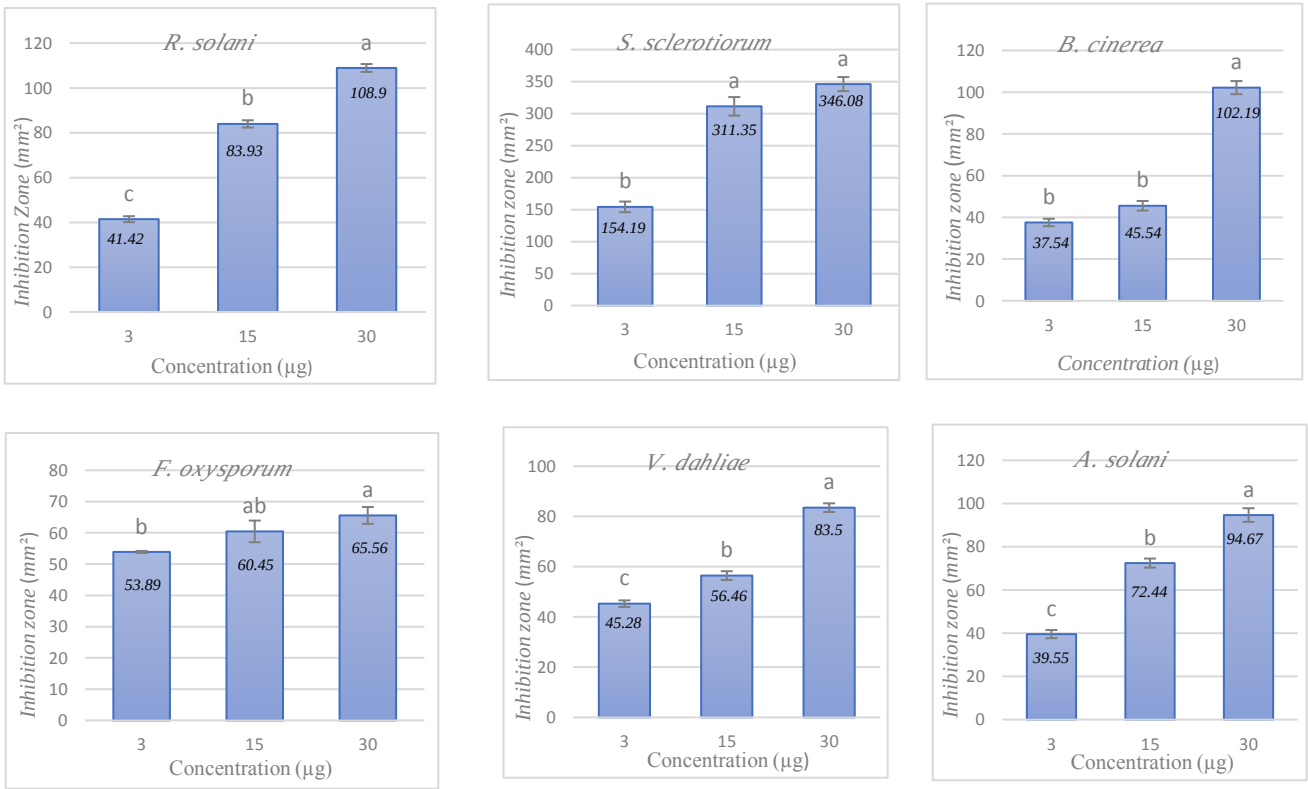


Figure 6. Radial diffusion assay of purified Osmotin. **A**: Representative bioassay Petri dishes. Inhibitory effect of Osmotin on fungal growth were observed as areas of lacking mycelial growth. 1, 2 and 3: Different concentrations; 3, 15 and 30 µg of the purified Osmotin, respectively, 4: Control (elution buffer), 5: Control (extracted protein from non-induced *Escherichia coli*). **B**: Average area (mm^2) of mycelial growth inhibition on different fungi coused by expressed Osmotin. Results represent the average and standart deviation of three experiments.

In *in vitro* disc diffusion assay, hyphal growth was decreased by increasing the concentration of the expressed Osmotin (1, 6 and 12 µg) (Figure 7).

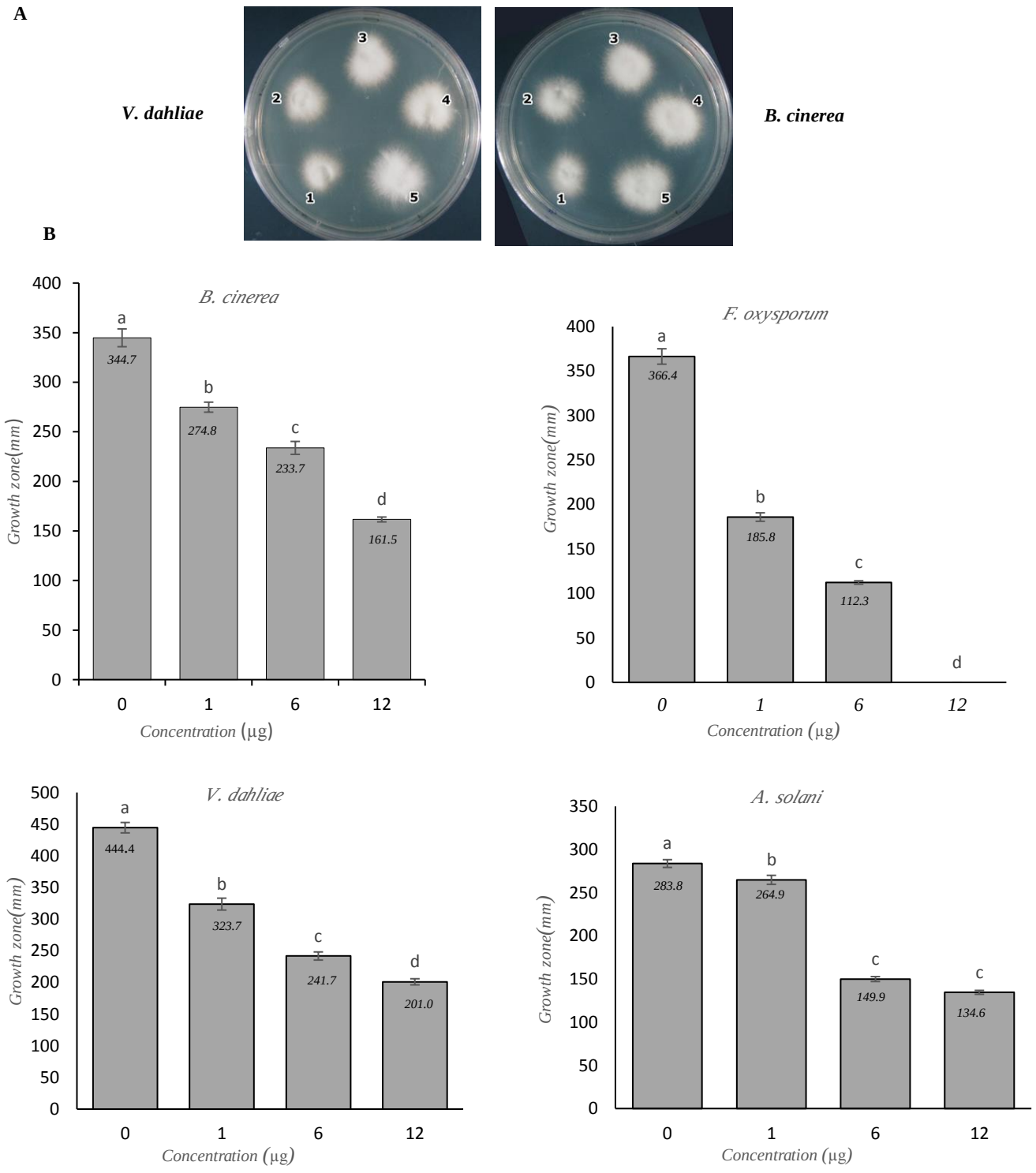


Figure 7. Disk diffusion assay. **A**: Representative bioassay Petridishes. Inhibitory activity of the Osmotin on pathogenic fungal growth. 1, 2 and 3: 12, 6 and 1µg of the purified Osmotin, respectively, 4: Control (elution buffer), 5: Control (extracted protein from non-induced *Escherichia coli*). **B**: Growth zone diameter of pathogenic fungal growth. Results represent the average and standart deviation of three experiments.

In spore germination assay 15 µg of the purified Osmotin was able to inhibit the germination of fungal spores (Figure 8). Comparison of the amount of Osmotin used in radial diffusion and spore germination assays indicates that the spore germination assay is more sensitive than the radial diffusion assay.

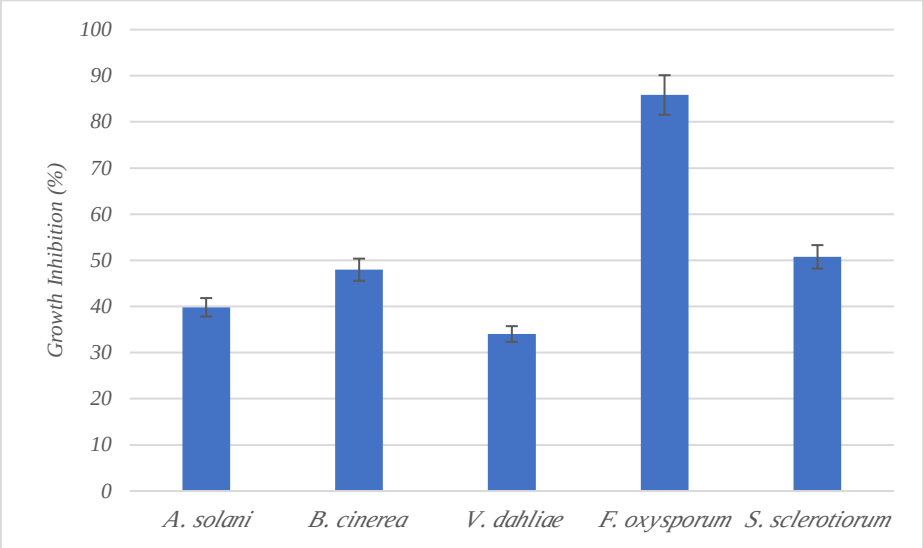


Figure 8. Growth inhibition of purified Osmotin on spore germination. Growth of different fungi in crude protein extracts from *Escherichia coli* Rozetta (DE3) containing Osmotin. After 96 hours of incubation, the absorbance of the reaction mixture (crude protein extract + spore suspension + PDB) was measured at 595 nm. Control (spore growth in the absence of Osmotin). Assay (spore growth in the presence of Osmotin). The percentage of growth inhibition was calculated as 100 × the ratio of the A595 of the control minus the A595 of the sample over the A595 of the control .The experiments were conducted three times.

The hemolysis assay used in this study has the advantages of being cheap, accessible, and simple to perform. In the hemolysis assay, we utilized 1% washed human red blood cells. These erythrocytes were incubated with 60 µg of pure Osmotin for a duration of 60 minutes at a temperature of 37 °C. Following the incubation period, we measured the optical density (OD) at 405 nm to quantify the amount of hemoglobin released, which serves as an indicator of hemolysis. Positive control (10% Triton X100) and negative control (phosphate-buffered saline, PBS, pH 7) were used in this assay. No significant hemolytic activity (less than 0.5%) was observed at a concentration of up to 60 µg of recombinant Osmotin.

Discussion

Over the past two decades, many researchers have conducted extensive studies on PR proteins (Sundar *et al*. 2002; Selitrennikoff 2001). In this study, the antifungal activity of an antimicrobial peptide was evaluated on fungal pathogens that are important for crop production. The coding region of the Osmotin signal peptideless mature protein was used for expression in *E. coli*. The pET26b (+) expression vector was chosen because it produced appropriate quantitative of protein in *E. coli*. The histidine-tag's presence had no impact on the inhibitory function of the expressed protein, as previously noted by Gianotti *et al.* (2006).

Escherichia coli. was chosen as the expression host due to its ease of handling, cost-effectiveness, and ability to produce proteins rapidly and in vast quantities, as stated by Makrides (1996). Protein expression is typically influenced by variations in codon usage (Gustafsson *et al*. 2004) which can be particularly challenging when rare codons used commonly in eukaryotes but infrequently in *E. coli* are present. Analysis of the coding sequence of the *osmotin* gene indicates that there are some rare

codons among the codons in the DNA sequence, necessitating the use of the *E. coli* Rosetta (DE3) strain to compensate for these differences. In this study, the Rosetta (DE3) strain was utilized to successfully express the *Osmotin* gene despite its inappropriate codon usage.

There are some reports indicating that signal peptides in the N-terminal of PR-5 preprotein affect the *E. coli* growth and hinder the expression of the protein (Singh *et al*. 1987; Low *et al*. 2012). Similarly, the introduction of a defensin gene from *Trichosanthes kirilowii* Maximowicz, along with a signal peptide, was not induced when using pET32a (+), as reported by Hu & Reddy (1997) .

Using the Taguchi method, the Osmotin protein was expressed optimally under conditions of 0.7 mM IPTG concentration and 6 hours of incubation at 37°C. Western blot analysis confirmed the existence of the expected band for the expressed protein. The purified and refolded protein was then tested for its antifungal activity against various native plant fungal pathogens, including *R. solani*, *B. cinerea*, *S. sclerotiorum*, *F. oxysporum*, *V. dahliae* and *A. solani*. Results from radial diffusion and spore germination assays indicated that an increase in the concentration of the expressed protein led to a decrease in the hyphal growth and spore germination of the fungi. Similar results were reported in a study of the antifungal activity of Osmotin (Tzou *et al*. 2011). Furthermore, Olli and Kirti (2006) found that heterologous expressed antimicrobial peptides in *E. coli* inhibited the mycelial growth of *R. solani*.

The preproprotein precursor usually contains a signal peptide at its N-terminus that aids in the transportation of the protein via the secretory pathway (Bol *et al*. 1990; Melchers *et al*. 1993). Additionally, it contains a carboxy-terminal extension that could be cleaved off either during or after transportation to the vacuole (Melchers *et al*. 1993; Liu *et al*. 1994; Sato *et al*. 1995). Bacterial expression systems, though they cannot fully eliminate signal peptides or correctly create all disulfide bonds found in eukaryotic proteins, are still a more economical and efficient alternative to other eukaryotic cell systems such as yeast, insect, or mammalian cells when it comes to express heterologous proteins. To enhance the production of active forms of Osmotin, this study employed an *E. coli* expression system and *in vitro* post-expression refolding. In order to express mature Osmotin in *E. coli*, to remove the signal peptide and carboxy-terminal extension from the prepro Osmotin ORF, PCR-engineering was utilized. The successful implementation of these methods resulted in high yields of active Osmotin. When proteins are produced in large amounts, exceeding approximately 30% of the total protein contents of the host cell, it is common to observe the formation of inclusion bodies, which are abundant within the insoluble protein fraction. Both inclusion bodies and protein precipitation are examples of protein aggregation, which can result in the presence of misfolded protein molecules (Pain 1994). Despite the formation of inclusion bodies as a result of overexpression of Osmotin in *E. coli*, the inclusion bodies were successfully repaired by procedures of denaturing and subsequent refolding. These processes resulted in the recovery of mature, biologically active conformations of Osmotin, which exhibited antifungal activity on phytopathogenic fungi. Several proteins with a similar structure to PR5 have been expressed in *E. coli* and have demonstrated biological activity (Hu & Reddy 1997; Newton & Duman 2000). In their study, Hu & Reddy (1997) showed that an Arabidopsis thaumatin-like protein, when expressed in *E. coli*, could be obtained as inclusion bodies, purified, and subsequently refolded into its mature form (Hu & Reddy 1997). The mature protein exhibited activity against specific phytopathogenic fungi. The investigation conducted by Newton *et al.* (2000) revealed that an Osmotin-like cryoprotective protein, when expressed in *E. coli* and directed to the periplasm, yielded high concentrations of active and soluble protein (Newton & Duman 2000). In contrast, expression of the protein in the bacterial cytoplasm resulted in the production of substantial amounts of insoluble protein. In this study, despite the use of *pel*B signal peptide, the expressed protein turned into inclusion body, maybe due to overexpression of the protein, which required denaturation and refolding.

Assessment of cytotoxicity plays a crucial role in

characterizing new compounds or materials that are intended for interactions within human biological systems *in vivo*. One important aspect of cytotoxicity evaluation involves examining the compound's ability to induce disruption of erythrocyte membranes, leading to the release of cellular content. This step is often an initial stage in assessing cytotoxicity and provides valuable information about the potential impact on cellular integrity. The hemo-compatibility of Osmotin was checked by testing the hemolytic activity, as it may have potential applications in antifungal drugs or transgenic plants for human use. The expressed protein displayed no hemolytic activity, demonstrating hemo-compatibility of Osmotin protein.

In this study, it was shown that the Osmotin, in a refolded form, demonstrates antifungal activity on six economical important native phytopathogens. Therefore, this gene, which is able to control the growth of these pathogens, has the potential to be utilized for the transgenic management of phytopathogenic fungi.

Acknowledgments

This research was supported by National Institute of Genetic Engineering and Biotechnology (NIGEB). We would also like to thank M.R. Zamani for helpful discussions.

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